REMARKS

Applicants respectfully request reconsideration and withdrawal of the finality of the Action mailed March 11, 2005. Applicants submit that the final rejection was premature because a new rejection was not necessitated by the amendment to the claims. The amendment did not add or remove a limitation that materially affected or changed the scope of the claims, because the claims already encompassed an attenuated *Salmonella* strain comprising the eukaryotic expression of a polypeptide in the host cell of a vertebrate to generate an immune response and act as a vaccine. In view of the remarks made herein, Applicants respectfully request that the finality of the Action under MPEP § 706.07(a) be withdrawn.

Claims 1-6, 9, 10 and 17-23 are pending. Claims 7 and 8 were previously canceled and claims 11-16 were previously withdrawn. The amendments to the claims are respectfully requested to be entered pursuant to 37 C.F.R. §1.116 to place the claims in better condition for allowance or appeal. Amendment of claims herein is made without abandonment of the original subject matter; Applicants reserve the right to pursue claims of the original or similar scope in a duly filed continuing application.

Claim 1 has been amended to recite "[a]n attenuated *Salmonella* strain comprising a eukaryotic expression vector comprising a eukaryotic promoter and a nucleic acid encoding a polypeptide, wherein said nucleic acid is under the control of said eukaryotic promoter, wherein the attenuation is suitable for a vaccination of a vertebrate, and wherein vaccination of said vertebrate with said attenuated *Salmonella* strain results in expression of said polypeptide by said vertebrate and generates an immune response by said vertebrate to said polypeptide." Support for the amendment to claim 1 is found throughout the specification and at page 3, lines 1-2; page 3, line 28 through page 4, line 18; pages 8-10; page 14, lines 14-29; and page 38 of the application as originally filed. Claims 9 and 23 have been amended as requested by the Examiner to clarify the recited gene. Support for the amendments to claims 9 and 23 is found at page 3, lines 12 through 17. Claim 17 has been amended to be consistent with the language of claim 1, from which it directly depends. Claim 18 has been amended to recite "IgG1, IgG2, and IgA antibodies." Support for the amendment to claim 18 is found at page 7, lines 7-26. Claims 11-16, previously withdrawn, are canceled herein to place the application in condition for allowance.

Thus, all claim amendments herein find basis in the original specification and no new matter has been introduced.

OUTSTANDING REJECTIONS

Claim 18 remains rejected and dependent claim 21 now stands rejected under 35 U.S.C. §112, first paragraph, as assertedly containing new subject matter.

Claims 1-6, 9, 10, and 17-23 were rejected under 35 U.S.C. §112, first paragraph, as assertedly lacking enablement.

Claims 1-6, 9, 10, and 17-23 were rejected under 35 U.S.C. §112, second paragraph, as assertedly being indefinite.

Claims 1, 2, 4-6, 9, 10, and 18-20 were rejected under 35 U.S.C. §103(a) as assertedly being anticipated by Branstrom et al. (U.S. Patent No. 5,824,538, filed September 6, 1995, hereinafter "Branstrom") in view of Stocker et al.(*Intern. Rev. Immunol.* 11:167-178, 1994; hereinafter "Stocker").

Claim 3 was rejected under 35 U.S.C. §103(a) as assertedly being anticipated by Branstrom as modified by Stocker as applied to claims 1 and 2, and further in view of Fouts et al. (*Vaccine* 13:1697-1705, 1995; hereinafter "Fouts").

PATENTABILITY ARGUMENTS

1. The rejections under 35 U.S.C. § 112, first paragraph, for new subject matter

While Applicants disagree with the Examiner for the reasons provided in the prior response, Applicants have amended claim 18 to delete the term "or" solely in order to expedite prosecution. The rejection of claim 18 and claim 21 dependent thereon at pages 4-5 of the Action are mooted by the amendment to claim 18 herein.

2. The rejections under 35 U.S.C. § 112, first paragraph, for lack of enablement

The Examiner has asserted at pages 5-6 of the Action that claim 1 lacks enablement because it allegedly does not include the subject matter that is essential to the practice of the invention. Applicants disagree. As Applicants have noted before, the difference between the claimed invention and the prior art is that the *Salmonella* vaccine contains an expression vector that permits expression of the antigen by the *eukaryotic host*, in contrast to

expression of the antigen by the *Salmonella* itself via a prokaryotic expression vector. Nothing more is needed to practice the invention.

Nevertheless, Applicants have clarified claim 1 solely in order to expedite prosecution. The nature of the eukaryotic expression vector (i.e. that it contains a promoter suitable for expressing a protein in eukaryotes, such promoter being operably linked to the nucleic acid encoding the antigen) has been clarified as suggested by the Examiner. Since the nature of an existing claim term has merely been clarified, such amendment does not alter the scope of the claim. The amendment to claim 1 also clarifies the meaning of attenuation (attenuated so that the strain is suitable for vaccination). Finally, Applicants have amended claim 1 to add a recitation of the function of the claimed strain (as a vaccine for generating an immune response in response to expression of the antigen by the vertebrate rather than expression of the antigen by the *Salmonella*).

Thus, claim 1 now recites that the attenuated *Salmonella* strain allows for the expression of the polypeptide by the host, not by the bacteria. Claim 1, as amended, clearly conveys to one of ordinary skill in the art that (1) the attenuated bacterial strain comprises a eukaryotic promoter and a gene ("nucleic acid encoding a polypeptide"); (2) a eukaryotic host (vertebrate) expresses the gene on genetic immunization with the strain ("wherein vaccination of said vertebrate with said attenuated *Salmonella* strain results in expression of said polypeptide by said vertebrate"); and (3) the immune response generated by the vaccine comprising the strain is due to the transfer of the gene and expression of the gene by the eukaryotic host ("generates an immune response by said vertebrate to said polypeptide").

Claim 1 as amended is believed to clearly convey the nature of the invention and thus the Examiner's rejections are moot.

3. The rejections under 35 U.S.C. §112, second paragraph

Applicants have amended claim 9, which was rejected for being assertedly confusing in the use of the terms "a gene (hly gene)" and "a gene (actA gene)" to recite the corresponding polypeptide, consistent with the terminology used in amended claim 1 The rejection of claim 9 at pages 6-7 of the Action is believed to be mooted by the amendment to claim 9 herein.

Applicants have amended claim 1, which was rejected for the use of the term "fragment," to delete the term and thus moot the rejection of claim 1 at page 7 of the Action.

Applicants have amended claim 23, which was rejected for the use of the phrase "a gene (hly gene) encoding a non-hemolytic truncated Listeria moncytogenes-literiolysin gene (hly gene)" to recite the corresponding polypeptide, consistent with the terminology used in amended claim 1. The rejection of claim 23 at page 7 of the Action is believed to be mooted by the amendment to claim 23 herein.

The rejection of claims 2-6, 9, 10, and 17-23, which depend directly or indirectly from claim 1, at page 7 of the Action is mooted by the amendment to claim 1 herein.

4. The rejections under 35 U.S.C. §103

Claims 1, 2, 4-6, 9, 10, and 18-20 were rejected under 35 U.S.C. §103(a) as assertedly being anticipated by Branstrom in view of Stocker. Branstrom disclosed an attenuated *Shigella* strain comprising an expression vector for delivering a mammalian expression plasmid into cells. The Examiner's reliance on Branstrom as the primary reference is misplaced because (1) Branstrom does not teach the subject matter of claim 1, which is an attenuated *Salmonella* strain containing a *eukaryotic expression vector* that is *effective for genetic immunization (i.e.,* wherein vaccination of said vertebrate with said attenuated *Salmonella* strain results in expression of a polypeptide by said vertebrate and generates an immune response by said vertebrate to said polypeptide*)*, and (2) Branstrom actually teaches away from the genetic immunization of the present invention by stating that:

> "Although we have no formal proof that release from the phagocytic vacuole into the cell cytoplasm by the bacteria is essential for DNA delivery, preliminary experiments with ***Salmonella typhurium***, an organism that reaches the cytoplasm only with difficulty, suggests **this organism is not an efficient DNA delivery vehicle**" (emphasis added).

See Branstrom at column 19, line 40, through column 20, line 4. Thus, Branstrom does not suggest the use of attenuated *Salmonella* for delivering DNA (a eukaryotic expression vector) into cells for the genetic immunization of vertebrates whereby the host expresses the antigen. Moreover, this statement suggesting that *Salmonella* is not useful for DNA delivery is repeated by the same authors in their parallel scientific publication [see Sizemore et al., *Science* 270:299-302, 1995 at page 301, right column, second paragraph (enclosed as **Exhibit A**)]. Thus, the cited art would not motivate or suggest to one of skill in the art to use *Salmonella* for the delivery of genes (DNA) into mammalian cells for the genetic immunization of vertebrates as is claimed in the instant application.

The secondary reference of Stocker does not correct any of the deficiencies of Branstrom. The Examiner asserted that Stocker disclosed that live oral vaccines of *S. typhi* were available, already in widespread use, and proven safe and reasonably effective for expression of a heterologous protein. *See* the Action at page 8. However, Stocker merely states that there is "one oral-route live vaccine, strain Ty21a, proven safe and reasonably effective, already in widespread use," (*see* Stocker at page 167). Stocker says nothing about the use of strain Ty21a for delivery of a *eukaryotic expression vector* comprising a nucleic acid encoding a polypeptide, under the control of a eukaryotic promoter, for genetic immunization of a vertebrate. Rather, Stocker describes the use of a chimeric flagellin as a vaccine and states that only repeated injection with live vaccine with flagella made of flagellin incorporating the heterologous protein epitope resulted in substantial antibody production. Thus, Stocker teaches one that vaccine (not the host) provides the antigen, teaching away from genetic immunization whereby the host expresses the antigen. *See* Stocker at page 176. Furthermore, Stocker does not describe oral immunization of humans with attenuated *Salmonella* strains that have been transformed with expression vectors like the type described in the claims of the instant application. In addition, there is no motivation or suggestion to combine Branstrom and Stocker. The Examiner has not shown where in the art such a combination is suggested. Moreover, even if the references were improperly combined, the combination does not teach Applicants' claimed invention because Branstrom is teaching away from the claimed invention. Accordingly, the Office has failed to establish a *prima facie* case of obviousness for the claimed subject matter in view of Branstrom and Stocker and, thus, the rejection of claims 1, 2, 4-6, 9, 10, and 18-20 under § 103(a) over Branstrom in view of Stocker has been overcome.

Claim 3 was rejected as assertedly being anticipated by Branstrom as modified by Stocker as applied to claims 1 and 2, and further in view of Fouts. Fouts assertedly discloses an attenuated *S. typhimurium* aroA SL 7207 vaccine strain specifically for expressing a heterologous antigen to elicit a heterologous antigen-specific immune response. See the Action at pages 8-9. However, Fouts does not disclose an attenuated *Salmonella strain* which comprises a *eukaryotic expression vector* that allows efficient expression of the heterologous gene in the eukaryotic host which has been immunized with the *Salmonella*. Fouts merely discloses an attenuated *S. typhimurium* aroA SL 7207 vaccine strain bearing a heterologous gene under the control of a prokaryotic promoter. The ability of an immunized eukaryotic host to express the gene is an important point of the instant application because that is the mechanism by which the genetic immunization occurs. Applicants' experimental data at pages

8-10 showed that the immune response generated by the vaccine was due to the *in vivo* transfer of the gene and expression of the gene by the mice, not due to the expression of the gene by the *Salmonella*.

Thus, Fouts does not disclose an attenuated *Salmonella* strain comprising a eukaryotic expression vector (under control of a eukaryotic promoter), and the secondary references of Stocker or Fouts do not correct the deficiencies of Branstrom as set out above. Thus, the combination of Branstrom, Stocker, and Fouts do not render obvious claim 3 and the rejection should be withdrawn.

In addition, Applicants are enclosing, as **Exhibit B**, a publication by Lowrie (*Nature Medicine* 4:147-148, 1998; hereinafter "Lowrie") that provides objective evidence that others in the art viewed the claimed invention as unexpected and surprising. The Lowrie article refers to the publication of Darji et al. [*Cell* 91:765-775 (1997); hereinafter "Darji"], which was authored by the inventors of the present application and discloses the same data as the present application. (Darji was enclosed with the last Response to an Office Action mailed December 10, 2004). *See* Lowrie at page 147, central column, second paragraph, wherein Lowrie states in referring to the work by Darji that:

> "the **unexpected and marked success of the salmonella DNA delivery vehicle** further challenges the perception that DNA vaccination is abnormal biology and is therefore inherently dangerous. **The success of salmonella is surprising** because – in contrast to shigella and invasive *E. coli*, both of which escape from the phagocytic vacuole to enter the cytoplasm in cells – this bacterium is believed to be retained within the phagolysosome, the normal fate of particles engulfed by phagocytes" (emphasis added).

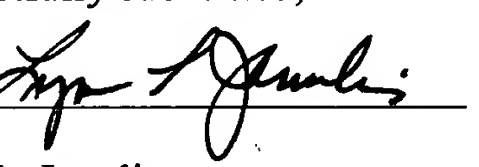
This publication by Lowrie demonstrates that one of skill in the art found the invention of the instant application, as described in Darji's publication, to be unexpected and unobvious. Thus, the rejection of claims 1-6, 9, 10, and 18-20 under 35 U.S.C. §103(a) for obviousness should be withdrawn.

CONCLUSION

For the foregoing reasons, each of claims 1-6, 9, 10 and 17-23 is believed to be in condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejections of the claims and to pass this application to issue.

Dated: June 13, 2005

Respectfully submitted,

By 

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mal growth factor, fibroblast growth factor, or angiotensin II all produce a rise in $[H_2O_2]$ with a correlation existing between the magnitude and duration of an increase in $[H_2O_2]$ and the level of tyrosine phosphorylation (*10*). These latter observations also strengthen the case that in many ways H_2O_2 fulfills the definition of an intracellular second messenger.

VSMCs appear to be unusual in their uptake of extracellular catalase. Certain cells secrete catalase, and the amount of catalase in serum increases in certain disease states (*14*). Thus, growth of VSMCs might be influenced by extracellular catalase in vivo. Recent epidemiological studies suggest a cardioprotective effect of antioxidants (*15*). Given that PDGF-induced VSMC migration and proliferation is thought to precipitate early atherogenic changes (*16*), one mechanism by which dietary antioxidants might protect against cardiovascular events is by a direct effect on H_2O_2-mediated signal transduction in VSMCs.

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17. Primary VSMCs were obtained from rat thoracic aorta by enzymatic digestion as described [J. H. Campbell and G. R. Campbell, *Vascular Smooth Muscle in Culture* (CRC, Boca Raton, FL, 1987), pp. 15–22] and were stimulated with PDGF-AB (5 ng/ml); DCF fluorescence was measured (6). Images were collected with a Leica Laser confocal scanning microscope, model TCD4. Relative DCF fluorescence was recorded on a scale from 0 to 256. In general, basal fluorescence averaged from 0 to 10 units, whereas stimulated fluorescence averaged from 60 to 200 units. Although DCF is oxidized by both H_2O_2 and hydroxyl radicals, the lack of fluorescence in PDGF-stimulated catalase-loaded cells suggests that the fluorescent signal after the addition of growth factor is predominantly derived from H_2O_2 (see Fig. 2E).
18. VSMCs deprived of serum for 3 days were stimulated with the indicated concentration of H_2O_2 or PDGF-AB (5 ng/ml) for 20 min, after which cells were harvested and lysed in RIPA buffer [50 mM tris-Cl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS]. Immunoprecipitation was done from 150 mg of unstimulated or stimulated cell lysate with aphosphotyrosine antibody (4G10, UBI). Immunoprecipitated proteins were divided in equal portions, and tyrosine-phosphorylated proteins from 50 mg of lysate were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (4 to 20% gels) and transferred to a nitrocellulose filter. When indicated, filters were probed with either an antibody to phosphotyrosine (RC20), a broadly reactive antibody to MAP kinase (erk1-CT, UBI), or an antibody to paxillin (P13520, Transduction Lab). Amounts of MAP kinase protein were detected from total cell lysates (10 μg per lane) by erk1-CT. Antibody binding was visualized by enhanced chemiluminescence (Tropix).
19. Adenoviral stocks were prepared and titered on 293 cells and used to infect VSMCs as described [R. J. Guzman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10732 (1994)]. Three days after infection, cells were deprived of serum for 3 days, then triplicate cultures were collected in buffer A [1× phosphate-buffered saline (PBS) (0.0067 M), 10 mM EDTA, 2% Triton X-100, and 0.5% deoxycholic acid] for measurement of enzymatic activity or in RIPA buffer for analysis with antibodies to phosphotyrosine.
20. Confluent VSMCs (1×10^6 cells), HUVECs [American Type Culture Collection (ATCC), Rockville, MD], and HeLa cells (ATCC) that had been deprived of serum were incubated with beef liver catalase (3000 U/ml, Boehringer Mannheim) for various times. After incubation, cells were washed twice in PBS, trypsinized, and lysed in buffer A. Catalase activity was assayed by the rate of decrease in absorbance at 240 nm by the method described by H. Aebi [*Methods Enzymol.* **105**, 121 (1994)].
21. Cells incubated with extracellular catalase (3000 U/ml) were collected, and triplicate cultures were exposed to proteinase K (1 mg/ml) in solution for various times. At the indicated time, cells were quickly sedimented and proteinase K diluted and removed. The cells were subsequently lysed in buffer A. Residual catalase activity was measured as described (*20*).
22. Confluent VSMCs with and without catalase loading were stimulated for 30 min with 10 mM SNP. Cells were subsequently lysed with 6% trichloroacetic acid (TCA). Lysates were neutralized, and equal amounts (2×10^5 cells) were used to determine amounts of cGMP by radioimmunoassay (Amersham) according to the manufacturer's recommendation.
23. Cells were stimulated with PDGF (5 ng/ml) for 20 min, and MAP kinase proteins were partially purified by phenylsepharose chromatography as described [S. Offermanns, *J. Immunol.* **152**, 250 (1994)]. Myelin basic protein (MBP) phosphorylation was used as an index of MAP kinase activity, where 100% activity represents the activity derived from PDGF-stimulated cells in the absence of catalase loading.
24. Confluent VSMCs were maintained in media without serum for 3 days and then, as indicated, stimulated with PDGF-AB (5 ng/ml). After 18 hours, cells in quadruplicate wells were incubated for 3 hours with [^{3}H]thymidine (1 mCi/ml), and TCA-precipitable counts were determined. If VSMCs were stimulated with PDGF in the presence of 0.5% serum, we noted effects on both basal and PDGF-stimulated thymidine incorporation; under serum-free conditions, effects were present only with PDGF stimulation. This was presumably because of the effects of catalase loading on residual growth factors present under 0.5% serum conditions.
25. VSMC migration was measured in response to PDGF stimulation in a mini-Boyden chamber as described [S. Biro *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 654 (1993)]. In the absence of PDGF stimulation, there was on average 10 VSMCs per high-power field.
26. A 200-mM stock of NAC was adjusted to pH 7.4 by NaOH, flash frozen, and stored in portions at −80° for up to 1 month. VSMCs were treated with the indicated amount of NAC for 8 hours before stimulation.

27. We acknowledge R. G. Crystal for providing the adenoviral constructs; M. Beaven, Y. F. Zhou, W. S. Kwon, R. Vemuri, and N. Epstein for helpful advice; S. G. Rhee and S. E. Epstein for critical review and helpful comments; and D. Koch for preparation of the manuscript. M.S. was supported by the Howard Hughes Medical Institute as an NIH Research Scholar.

1 June 1995; accepted 21 August 1995

Attenuated *Shigella* as a DNA Delivery Vehicle for DNA-Mediated Immunization

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Direct inoculation of DNA, in the form of purified bacterial plasmids that are unable to replicate in mammalian cells but are able to direct cell synthesis of foreign proteins, is being explored as an approach to vaccine development. Here, a highly attenuated *Shigella* vector invaded mammalian cells and delivered such plasmids into the cytoplasm of cells, and subsequent production of functional foreign protein was measured. Because this *Shigella* vector was designed to deliver DNA to colonic mucosa, the method is a potential basis for oral and other mucosal DNA immunization and gene therapy strategies.

Direct DNA-mediated immunization is an exciting new approach to vaccine development (*1*). We chose to exploit the ability of *Shigellae* to enter epithelial cells and escape the phagocytic vacuole as a method for directing plasmid DNA to the cytoplasm of the host cell for protein synthesis and processing for antigen presentation (*2*). To attenuate the *Shigella* vector, we made a deletion mutation in the *asd* gene encoding aspartate β-semialdehyde dehydrogenase, an essential enzyme that is required to synthesize the bacterial cell wall constituent

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diaminopimelic acid (DAP) (3, 4). The resultant 15D construct, a Δ*asd* isolate of *Shigella flexneri* 2a strain 2457T, was able to maintain the eukaryotic expression vector pCMVβ (5) in the absence of antibiotic-selective pressure. The plasmid pCMVβ expresses *Escherichia coli* β-galactosidase under the control of the immediate early promoter and enhancer from the human cytomegalovirus (CMV) in mammalian cells; this permitted easy analysis of mammalian cell–mediated gene expression after delivery.

Strain 15D was screened to ensure that the large plasmid that is essential for bacterial invasion of mammalian cells had not been lost during the genetic manipulations. Immunoblots verified that the strain continued to express the invasion-associated IpaB and IpaC polypeptides (6) and thus showed no loss of the invasion plasmid. To confirm earlier observations, we tested 15D and 15D(pCMVβ) for the ability to invade cultured baby hamster kidney (BHK) cells with and without DAP supplementation during the 90 min allowed for invasion (7–9). Examination by light microscopy of fixed and stained chamber slides revealed that in the absence of DAP, 15D and 15D(pCMVβ) entered 13% and 10% of the cultured BHK cells, respectively. By contrast, 33% (15D) and 29% [15D(pCMVβ)] of the BHK cells contained bacteria when DAP was present during the invasion step. Although both constructs were able to invade BHK cells, the addition of DAP during the invasion step increased the number of BHK cells infected and the number of viable bacteria recovered (9).

To test the ability of 15D to deliver plasmid DNA, we followed intracellular bacterial viability and β-galactosidase activity (Fig. 1) over a 48-hour time course (8, 10). Initially, 1×10^7 to 3×10^7 viable bacteria of each strain were recovered from monolayers of BHK cells with no detectable β-galactosidase activity in cell extracts. No β-galactosidase activity could be detected in bacterial extracts that were equivalent to the total number of bacteria added. At each assay point, a loss of 1 to 1.5 log units of viable bacteria occurred with no notable difference between strains 15D and 15D(pCMVβ). However, at both the 24- and 48-hour assay points, increasing units of β-galactosidase activity were readily detected in extracts of BHK cells infected with 15D(pCMVβ). The detected β-galactosidase activity did not result from expression within the bacteria because, although no activity was measured at the first two assay points, large numbers of viable bacteria were present. In addition, an isolate of 15D(pCMVβ) that did not express IpaB and IpaC (as measured by immunoblotting) was unable to bring about β-galactosidase activity at the 24-hour assay point.

Infected monolayers of BHK cells were immunostained to examine β-galactosidase expression within individual cells (Fig. 2) (8, 11). No intracellular immunostaining was observed in monolayers infected with

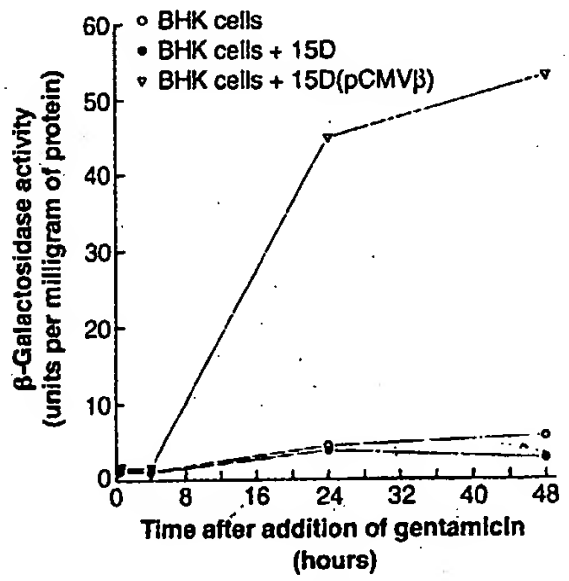

Fig. 1. Strain 15D was used as a carrier to deliver pCMVβ, a mammalian DNA expression plasmid, to BHK cells. β-Galactosidase activity (in units per milligram of protein) was determined for BHK cells alone (○), BHK cells infected with 3×10^9 15D (●), and BHK cells infected with 1×10^9 15D(pCMVβ) (▽). Determinations of β-galactosidase activity were made on an estimated 0.5×10^7 cells. Bacteria were grown as described (8). In this assay, DAP (50 μg/ml) was added to concentrated bacterial suspensions before these suspensions were added to flasks of semiconfluent BHK cells (~1×10^7 cells). At the indicated times, BHK cells were removed by trypsinization and washed in phosphate-buffered saline. A portion of the cell suspension was lysed with a 0.2% Triton X-100 solution, diluted, and plated on TSA Congo red DAP plates to determine the number of viable bacteria. β-Galactosidase activity was measured in the remaining cell extract by a standard biochemical assay (10) [units of β-galactosidase = 380 × OD_{420}/time (in minutes)]. β-Galactosidase activities were standardized to 1 mg of total protein, as determined with a BCA* protein assay kit (Pierce).

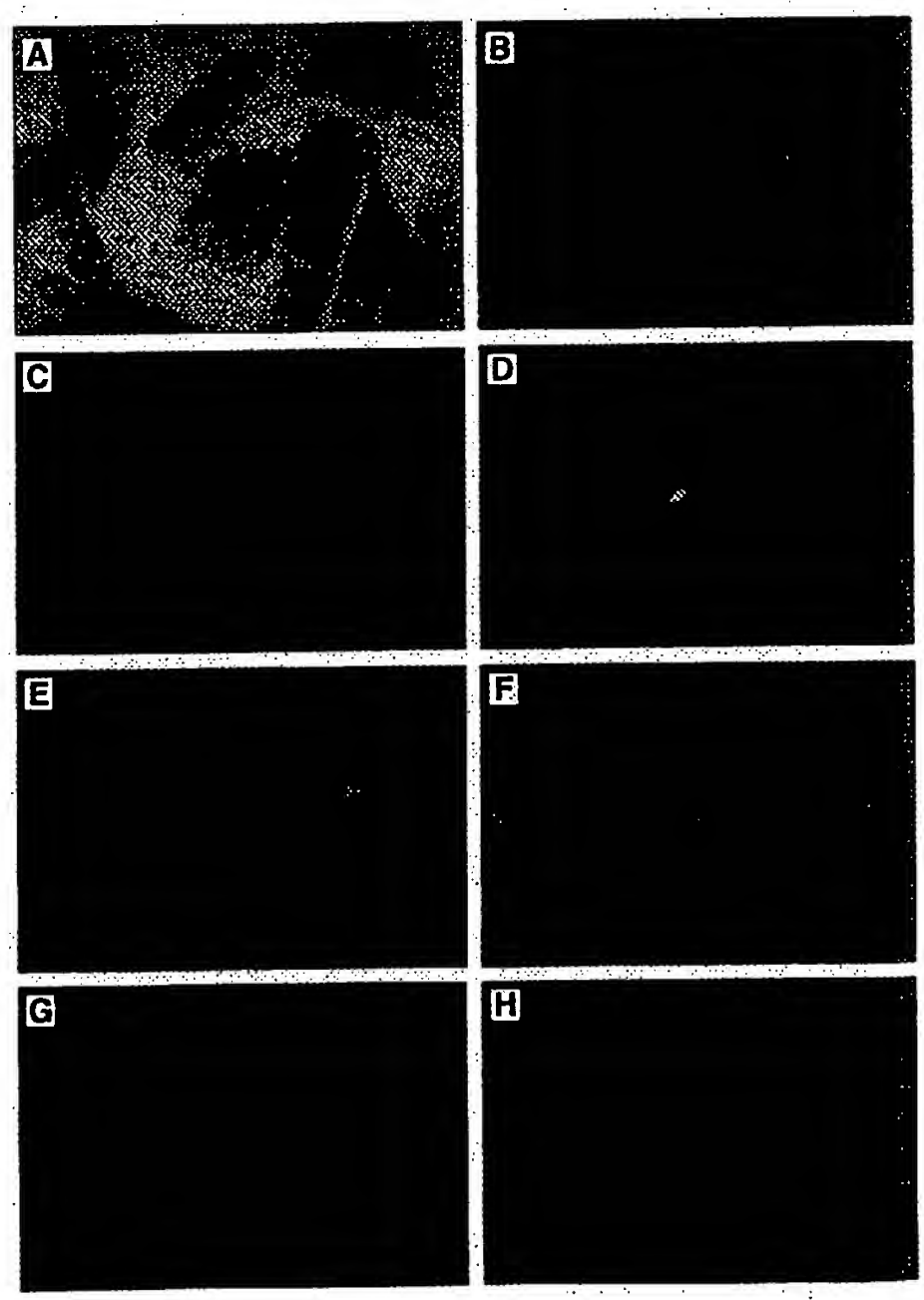

Fig. 2. Intracellular immunostaining to detect expression of β-galactosidase within BHK cells infected with 15D or 15D(pCMVβ). (**A**) A Leukostat-stained BHK monolayer infected with 15D(pCMVβ) 30 min after the addition of gentamicin-containing medium. (**B** through **H**) Immunostained infected BHK cells after the addition of gentamicin-containing medium. (B) 15D(pCMVβ), 30 min; (C) 15D, 4 hours; (D) 15D(pCMVβ), 4 hours; (E) 15D(pCMVβ), 24 hours; (F) 15D(pCMVβ), 48 hours; (G) 15D, 48 hours; (H) BHK cells alone. Three wells of a four-well chamber slide of BHK cell monolayers infected with 15D or 15D(pCMVβ) were immunostained to detect β-galactosidase expression (8, 10, 11). At the indicated times, washed monolayers were fixed in phosphate-buffered 4% paraformaldehyde for 5 min and then blocked with 3% goat serum (Gibco-BRL) in HBSS for 30 min. BHK cells were then permeabilized for 1 min with HBSS containing 0.1% saponin (Sigma). A monoclonal antibody to β-galactosidase (Sigma) was diluted 1:2000 in HBSS containing 0.1% saponin and applied for 30 min at 37°C in a humidified chamber. Fluorescein isothiocyanate–conjugated secondary antibody to mouse IgG (Fc-specific, Sigma) was diluted 1:32 and applied for 30 min at room temperature. Between each step, chamber slides were washed extensively with HBSS containing 0.1% saponin. A final wash step of HBSS alone was used to close permeabilized cells. Fluorescent images were visualized with a Nikon Microphot with epifluorescence attachment or with an Olympus VAN04-S with fluorescence attachment. Original magnifications, ×312.5 (A); ×62.5 (B through H).

either strain at the 30-min assay point (Fig. 2B). Only slight intracellular immunostaining was detected at the 4-hour assay point in monolayers infected with 15D(pCMVβ) (Fig. 2, C and D). By the 24- and 48-hour assay points, positive immunostaining of several cells per field was observed in monolayers infected with 15D(pCMVβ) (Fig. 2, E and F). Staining throughout the cytoplasm suggested that the plasmid DNA had been released from the bacterium into the cell cytoplasm, leading to transcription and translation by the mammalian cell. Immunostained cells also appeared to be rounded, possibly because of the presence of a large quantity of β-galactosidase protein. As measured by fluorescence-activated cell sorter (FACS) analysis, 1 to 2% of 5000 15D(pCMVβ)-infected BHK cells expressed β-galactosidase at the 24-hour assay point (*8*, *10*).

Visual examination of Leukostat-stained chamber slides of 15D(pCMVβ)-infected BHK cells indicated that 28% of the cells contained one to five visually intact bacterial cells, with 1.7% containing five bacteria (Table 1). Four hours after gentamicin treatment, 26% of the cells contained visually intact bacteria, with <1% of the cells containing four bacteria. Therefore, invasion with one to five bacteria was required for foreign gene expression. Because pCMVβ is a 7164–base pair plasmid that occurs in ~500 copies per bacterial cell, each bacterium is estimated to contain $\sim 3.93 \times 10^{-9}$ μg of DNA. Thus, intracytoplasmic delivery of no more than 4×10^{-9} to 20×10^{-9} μg of DNA by *Shigella* was sufficient for expression of β-galactosidase.

To demonstrate that gene delivery was not restricted to BHK cells, we infected murine P815 cells that express H-2^d class I major histocompatibility complex (MHC) molecules with 15D(pCMVβ). As shown in Table 2, 56.25 units of β-galactosidase activity were detected in lysates from P815 cells infected with 15D(pCMVβ). Further experiments will be necessary to determine whether these cells can present *Shigella*-delivered DNA-encoded foreign antigens in the context of class I.

Studies of the ability of 15D to deliver plasmid DNA in vivo have begun in two small animal models, the guinea pig keratoconjunctival and murine intranasal models, which are used to study *Shigella* pathogenicity and immunobiology (*12*, *13*). To determine whether 15D could deliver pCMVβ to the ocular surface of the guinea pig eye, we stained corneas for β-galactosidase activity and visually examined them at various times after inoculation (*12*). Varying amounts of staining were observed in the outer region of the cornea near the sclera of the right eyes that received 15D(pCMVβ), except those from day 8, in which staining was detected in only one of three corneas. Several areas typical of the staining observed in corneas that received 15D(pCMVβ) are shown in Fig. 3B. No apparent endogenous β-galactosidase activity was detected in eyes inoculated with 15D. Histology experiments will be needed to examine in greater detail the percentage of cells and cell type(s) invaded by 15D(pCMVβ) and those staining positive for β-galactosidase. In an initial experiment, spleen cells from intranasally inoculated BALB/c mice showed a moderate proliferative response to β-galactosidase protein (2.5 μg/ml) (*13*, *14*). The stimulation index (*14*) was 3.6 when the inoculum was supplemented with DAP compared with 2.1 in the absence of DAP. Although preliminary, these experiments indicate that bacteria can be used to deliver plasmid DNA in vivo.

Our method for delivering functional DNA inside cells need not be restricted to *Shigella* because the invasion genes used by *Shigella* can be inserted into other bacteria such as *E. coli* (*15*). Likewise, other bacteria such as *Listeria* are able to invade cells and break out of the phagocytic vacuole into the cytoplasm (*16*). Although we have no formal proof that such a release of bacteria from the phagocytic vacuole into the cell cytoplasm is essential for DNA delivery, preliminary experiments with *Salmonella typhimurium*, which reaches the cytoplasm only with difficulty, suggest that this organism is not an efficient DNA delivery vehicle (*17*).

Any bacterial DNA delivery system will need to strike a balance between cell inva-

Table 1. Percentage of BHK cells infected and number of bacteria per BHK cell, as shown by microscopic examination. Chamber slides and bacteria were prepared as described (*8*). At least 400 BHK cells of each group were examined.

Elapsed time (hours)	BHK cells infected (%) (mean)	Bacteria per infected BHK cell (mean ± SD)	Number of BHK cells containing 1 to 6 bacteria						
			1	2	3	4	5	6	Total
			Strain 15D						
0.5	39	1.84 ± 1.2	96	47	14	14	3	3	177
4	36	1.68 ± 0.94	106	36	13	5	0	1	161
24	3.7	1	17	–	–	–	–	–	17
48	2.2	1	10	–	–	–	–	–	10
			Strain 15D(pCMVβ)						
0.5	28	1.35 ± 0.72	76	29	7	5	2	0	119
4	26	1.40 ± 0.74	95	16	4	1	0	0	116
24	3.3	1	14	1	–	–	–	–	15
48	3.8	1	18	–	1	–	–	–	19

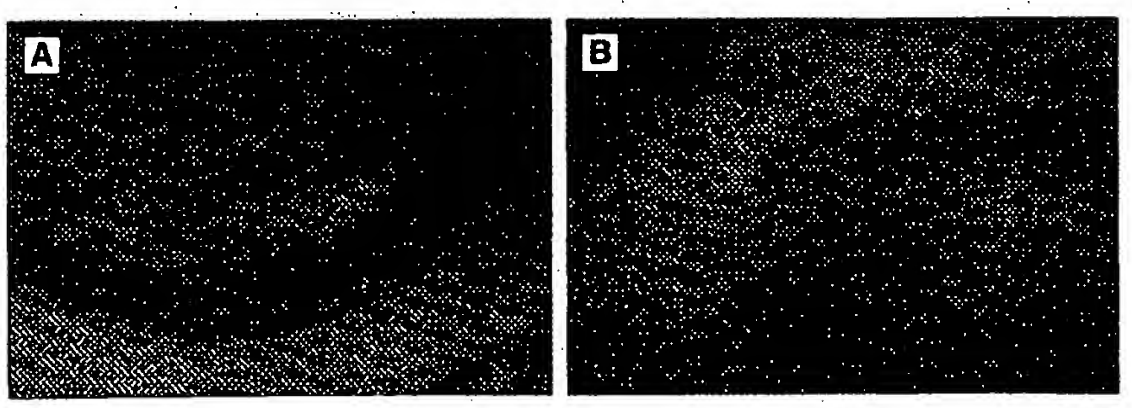

Fig. 3. Ability of 15D to deliver pCMVβ to ocular tissue. (**A**) Left cornea (15D) and (**B**) right cornea [15D(pCMVβ)], 48 hours after ocular inoculation. Arrowheads indicate areas of β-galactosidase staining.

Table 2. β-Galactosidase activity in P815 cells after infection with 15D(pCMVβ). Bacteria used to infect P815 cells were grown as described (*8*). After addition of the bacterial cultures containing DAP to the nonadherent P815 cells cultured in six-well plates, the plate was spun at 500*g* for 5 min. Bacteria and P815 cells were allowed to interact for 90 min. The cells were then extensively washed with DMEM and resuspended in DMEM containing gentamicin (100 μg/ml) for a 1-hour incubation at 37°C in the presence of 5% CO_2. The cells were again extensively washed and resuspended in DMEM containing gentamicin (20 μg/ml) for overnight culture at 37°C in the presence of 5% CO_2. β-Galactosidase activity and protein concentrations were determined at 24 hours as described (*8*, *10*).

Source	β-Galactosidase (units per milligram of protein)
P815 cells	3.04
P815 cells + 15D	5.62
P815 cells + 15D(pCMVβ)	56.25

sion (with its subsequent reactogenicity) and efficiency of delivery. In the case of *Shigella*, the genes responsible for invasion also cause invasion and apoptosis of macrophages, followed by inflammation (*18*). We constructed a *Shigella* strain that was completely unable to divide in the absence of DAP. Determination of the safety of this strain awaits human trials. Preliminary experiments with a guinea pig keratoconjunctivitis challenge model indicate that a two-dose immunization regimen followed by a challenge with virulent *Shigella* 3 weeks later gave 100% protection (*12*, *14*). These results demonstrate that this highly attenuated strain, which is capable of DNA delivery, functions well in vivo.

The bacterial DNA delivery system described here has several advantages for certain applications. Delivery of DNA-encoded antigens to the mucosal immune system should permit mucosal immunization simultaneously with multiple antigens that (i) can be directed for class I presentation, class II presentation, or both; (ii) can stimulate T helper cells (T_H1 or T_H2); and (iii) can be secreted while maintaining the proper folding and conformational epitopes for immunoglobulin A (IgA) and immunoglobulin G (IgG) antibody production. Diseases that may be especially responsive to this approach include diarrheal diseases such as rotavirus, gastrointestinal diseases such as the ulcer-causing *Helicobacter pylori*, and sexually transmitted disease agents such as human immunodeficiency virus, *Neisseria gonorrhoeae*, and human papilloma virus. Suppression of autoimmunity through manipulation of immune tolerance mechanisms in the gut has been demonstrated (*19*), and if such a technique proves to be generally applicable, it should also be amenable to our approach.

Perhaps the greatest advantage of bacterial delivery of DNA for vaccination and for potential gene therapy and replacement is the ease and acceptability of oral and other forms of mucosal delivery. Likewise, because no DNA purification is required for this type of DNA vaccination (which is essentially a live, attenuated bacterial vector), vaccines can be produced for the cost of fermentation, lyophilization, and packaging. Therefore, this type of vaccination may represent, at least in part, a solution to the costs and difficulties inherent in the production and development of current vaccines.

Aside from the practical applications of bacterial DNA delivery, the relatively efficient ability of *Shigella* to transfer functional DNA containing a eukaryotic promoter into mammalian cells leads to speculation concerning the potential role of such a mechanism in evolution. Plasmids for vaccine use are designed so as to minimize the possibility of chromosomal integration, but in nature this may not be the case.

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8. The general assay used for these studies was adapted from those described previously for *Shigella* plaque analysis (*6*, *7*). A single Congo red–binding positive colony (denoting the expression of plasmid-encoded *Shigella* virulence determinants) of each strain was used to inoculate overnight LB broth cultures containing DAP (50 μg/ml) (for 15D) or DAP plus ampicillin (250 μg/ml) [for 15D(pCMVβ)]. Overnight cultures were diluted 1:50 and grown to approximately mid–log phase in the presence of DAP. For chamber slide assays (Nunc) and 24-well plate assays, 200 μl of washed bacteria in Hanks' balanced solution (HBSS) with or without DAP (50 μg/ml) was added to three wells of semiconfluent BHK cells (1×10^5) at ~100:1. For flask assays, bacteria were grown as described, except that mid–log phase cultures were concentrated 10-fold and 2 ml of the bacterial solution was added to a flask of BHK cells (~1×10^7). In all assays, bacteria were allowed to interact with the BHK cells in this minimal volume for 90 min at 37°C in the presence of 5% CO_2. Nonadherent bacteria were removed by extensive washes with HBSS. Extracellular bacteria were then killed by the addition of Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (BioWhittaker) and gentamicin (50 μg/ml). For plating assays, cells were lysed with a 0.2% Triton X-100 solution and appropriate dilutions were plated on tryptic soy agar (TSA) Congo red DAP plates for determination of viable bacterial counts. At the indicated times, the chamber slides were extensively washed, fixed, and immunostained or stained with a Leukostat set (Fisher) before light or fluorescent microscopy. An Instat statistical program (Graphpad, San Diego, CA) was used to calculate means, SDs, and SEs. All data presented are representative of experiments performed two or more times.
9. The ability of a *Δasd* derivative of *S. flexneri* 2a strain 2457T to invade mammalian cells in culture was assayed to examine the requirement of DAP during the adherence and invasion step. Bacterial solutions with or without DAP were allowed to interact with BHK cells for 90 min, washed extensively, and then treated with gentamicin-containing media for 30 min before plating. In the absence of DAP, 1070 ± 404 (15D) and 1095 ± 332 [15D(pCMVβ)] viable bacteria were recovered, versus $8.2 \times 10^4 \pm 1 \times 10^4$ (15D) and $8.6 \times 10^4 \pm 3.5 \times 10^4$ [15D(pCMVβ)] when DAP was present [mean ± SE; $P = 0.024$ (Mann-Whitney test)].
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20. We thank C. Hammack, J. Grove, R. Curtiss III, E. Oaks, A. Hartman, and individuals in the Division of Medical Audio Visual Services at Walter Reed Army Institute of Research for providing materials or technical expertise. The views expressed in this report are those of the authors and not those of the U.S. Department of Defense.

6 June 1995; accepted 14 September 1995

DNA vaccination exploits normal biology

A live vaccine strain of salmonella can itself be used as a highly effective vehicle for oral delivery of DNA vaccines.

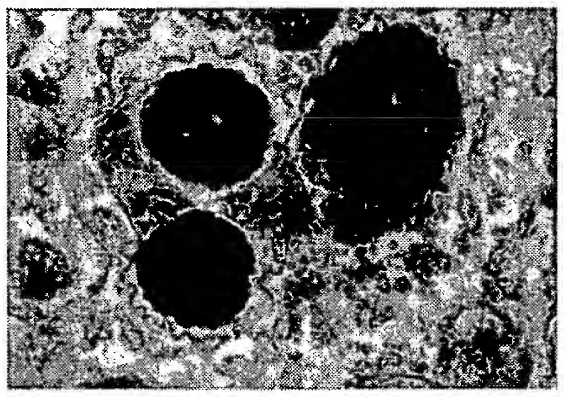
DOUGLAS B. LOWRIE

HOWEVER UNLIKELY IT may have seemed at first sight, there is now abundant evidence that DNA vaccines work[1]. The essence of this approach is that DNA encoding the antigen of interest is introduced directly into tissues and cells of the body, which then synthesize the antigen. DNA vaccination has the attractions of versatility, simplicity and potentially economy, but this is new science and there are still reservations. For example, very small amounts of antigen are produced and the vaccine DNA might integrate with nuclear DNA to generate tumors. Will DNA vaccines be effective enough and will they be safe enough for general use in man? A recent paper by Darji *et al.*[2] in *Cell* describes a novel way of enhancing the efficacy of DNA vaccines that simultaneously minimizes safety concerns.

The idea is that instead of growing the DNA vaccine as a plasmid in *Escherichia coli*, purifying the plasmid and administering it directly, the plasmid is grown in a mutant strain of salmonella that cannot grow *in vivo* and the transfected salmonellae are taken as an oral vaccine. The live attenuated bacteria carry the DNA through the stomach, then through the M cells that cover the Peyer's patches (lymph nodes) of the gut. From there the salmonellae enter macrophages and dendritic cells—the antigen presenting cells of the immune system—where they die because of their mutation, liberating multiple copies of the DNA vaccine right where they are needed, inside the phagocytes.

Darji and colleagues engineered the live attenuated aroA auxotrophic mutant of *Salmonella typhimurium* to carry either of two genes (under the control of a eukaryotic promoter) that encode virulence factors of *Listeria monocytogenes*. They then fed their oral DNA vaccine to mice. The salmonellae crossed the gut wall, presumably via the lymph nodes, and invaded phagocytic cells, their normal host cell. The subsequent systemic immune responses to the DNA-encoded antigens expressed by the phagocytes were remarkably strong and the response to one of them (a fragment of listeriolysin) protected against a lethal challenge with the *L. monocytogenes* pathogen.

Using bacteria in this way to carry DNA vaccines into antigen presenting cells is not a totally new idea as it has also been shown to work with auxotrophic mutants of shigella and invasive *E. coli* as the carriers[3,4]. However, in contrast to these organisms, salmonella has the major attraction that mutants are already in use as live vaccines in man and animals[5]. This should shorten the road to the clinic.



Invasion of a mouse peritoneal macrophage by *Salmonella typhimurium*. Macrophages were pre-loaded with ferritin to label their lysosomal contents and were then allowed to phagocytose salmonellae. Bacteria can be seen (together with ferritin) in two membrane-bound phagocytic vesicles (phagolysosomes) within a peritoneal macrophage.

Perhaps as importantly, the unexpected and marked success of the salmonella DNA delivery vehicle further challenges the perception that DNA vaccination is abnormal biology and is therefore inherently dangerous. The success of salmonella as a vector is surprising because—in contrast to shigella and invasive *E. coli*, both of which escape from the phagocytic vacuole to enter the cytoplasm in host cells—this bacterium is believed to be retained within the phagolysosome, the normal fate of particles engulfed by phagocytes (see figure). Microinjection shows that plasmid DNA that is introduced into host cell cytoplasm can readily enter the nucleus (by undefined mechanisms). This is a necessary step before the encoded antigens can be transcribed into mRNA and subsequently expressed. But for DNA entering host cell cytoplasm by phagocytosis the process is normally less efficient by several orders of magnitude. Contrast, for example, the microgram amounts of DNA needed for vaccination by intradermal injection with the nanogram amounts needed when a gene gun is used to introduce the DNA directly into cells[1]. So why is the phagocytosed salmonella vehicle so effective?

Hitherto, the primary interest in transgenic salmonella vaccines has been in their efficacy in generating immunity to foreign antigens that are expressed by the salmonella themselves under the control of bacterial promoters[5]. So Darji and co-workers decided to formally demonstrate that the macrophages, rather than the salmonella, were producing the antigen in their system. Although antigen expression from the plasmid was placed under the control of a eukaryotic promoter, conservation of regulatory elements between phyla can sometimes result in low-levels of gene expression in unlikely circumstances. However, none of the three antigens tested were secreted by the bacteria, which would be a prerequisite for the combination of strong responses of cytotoxic T cells, type 1 helper T cells and antibody that were observed. Furthermore, immunization with salmonellae that produced 100-fold higher amounts of antigen under the control of bacterial promoters produced a very inefficient immune response. Hence these experiments make it unlikely that bacterial production of the antigens contributed significantly to immunogenicity.

At this stage we simply do not know how plasmid DNA ever gets from a phagolysosomal vesicle into the nucleus of a phagocytic cell in a functional state, still less do we know how the salmonellae enhance this process. We cannot exclude the possibility that a minor proportion of phagocytic vesicles break down to discharge their contents into the cytoplasm, or that endosomal leakage and direct transport also occur. Darji *et al.* established that the overall process, measured as production of encoded protein, is much more efficient in primary macrophage cultures than in macrophage cell lines. This suggests that it is a property of mature differentiated cells.

Indeed they found that a remarkably high proportion of peritoneal macrophages expressed antigen from the plasmid after intraperitoneal injection of the salmonella vector. Expression appeared to continue long after the bacteria had disappeared. The intra-peritoneal route is totally ineffective for naked DNA vaccination and it is possible that the salmonella vehicle is so efficient simply because each bacterium delivers hundreds of plasmid copies into each phagolysosome and it is not necessary to postulate other mechanisms such as enhanced leakage or transport.

Consistent with efficient delivery into the phagolysosome—rather than enhanced egress to the nucleus—being the key to salmonella's success, there are reports suggesting that diverse inert biodegradable carrier particles containing plasmid DNA can also make highly effective oral DNA vaccines. Such particles have been prepared either by allowing anionic lipids and DNA to condense in the presence of calcium to form complexes termed cochleates[6] or by mixing DNA with poly-(lactide-co-glycolide) (from which biodegradable sutures are made)[7]. Remarkably, the trapped DNA is not only protected from enzymic degradation and acid hydrolysis in the stomach, it is also carried through M cells (if the particles are of a suitable size) and is delivered to macrophages and dendritic cells by a route that exactly parallels that taken by salmonella.

From these initial reports, oral DNA vaccines seem to be very effective. It follows that there may be a normal process whereby large segments of intact DNA trapped in particles get from the gut contents, through Peyer's patches, into the nuclei of antigen presenting cells. The DNA content of the gut is huge and includes DNA from the normal microbial flora and perhaps from food particles. Of course, linear DNA fragments are more susceptible than closed circular plasmid DNA to enzymic degradation and the likelihood of encoded genes being expressed is small, perhaps insignificant, even for DNA that bears eukaryotic regulatory elements. (Could we really be receiving genetic immunization from our food?) However, the very existence of the pathway from gut to phagocyte nucleus suggests that one of the main safety concerns over DNA vaccines—oncogenesis caused by random integration of foreign DNA into the nucleus—has already been extensively tested by normal gut biology. Considered separately, both oral vaccine delivery and DNA vaccination are especially attractive for vaccinating against Third World diseases, for reasons of simplicity and economy. How much more attractive then are they likely to be in safe and effective combination? The future for oral DNA vaccines looks bright.

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tPA: A neuron buster, too?

Studies in knockout mice suggest that the clot busting drug tPA harms neurons; experience in acute ischemic stroke patients says otherwise (pages 228–231).

GREGORY J. DEL ZOPPO

Because strokes occur predominantly in the elderly, there is little genetic pressure on humans to evolve an efficient way to favorably handle the resulting damage caused by reduced blood flow (ischemia) in the brain. This has led to reliance upon animal models, including knockout mice, to examine the mechanisms underlying brain injury and to develop interventions that will minimize injury after cerebral ischemia[1]. From such important work it has been found that various plasminogen activators—proteases that convert plasminogen to plasmin, which then lyses blood clots—are sequestered in brain tissue[2], microvessels[3], and neurons[4,5]. In the clinic, recombinant human tissue plasminogen activator (rtPA) has been shown to benefit selected acute ischemic stroke patients if given within three hours of the onset of symptoms[6].

On page 228 of this issue, Wang *et al.*[7] report that following a stroke, mice deficient in tPA show a significant reduction in infarct size compared to wild-type animals. The investigators blocked the middle cerebral artery (MCA) of normal and tPA deficient mice (in order to simulate a stroke) and then measured neuron and tissue responses to the reduced blood flow. They then infused recombinant human rtPA into animals during the period of focal ischemia and found a significant increase in infarct volume in both tPA null and wild-type control mice. They conclude that rtPA causes injury to neurons and should be used cautiously in human stroke victims.

This report raises two issues central to the treatment of stroke patients: (1) how do neurons, and presumably their surrounding tissue, respond to ischemic injury, and (2) how can findings in a small animal model be applied to the human patient? Recent experiences with recombinant plasminogen activators in stroke patients (given intra-arterially[8] or intravenously[6,9] within the first moments of focal cerebral ischemia) demonstrate that they significantly improve clinical outcome[6] by dissolving blood clots and increasing the likelihood of opening the occluded arteries that cause stroke[8]. Given that rtPA in one recent clinical study was responsible for a significant increase in the number of patients displaying improvements in disabilities[6], the report of Wang and colleagues demands our attention.

The current findings of Wang *et al.* are supported by previous results that they obtained using the same tPA knockout mice[4,5]. They found that the hippocampal neurons of tPA deficient mice were relatively resistant to seizure activity induced by excitotoxins such as kainate[4]. Administering rtPA to null and wild-type mice induced neurodegeneration in response to excitotoxins in the CA1–CA3